CAPRINE CARDIAC SARCOPLASMIC RETICULUM : EFFECT OF SIMULATED ISCHEMIA ON MEMBRANE STRUCTURE AND Ca^{2+} -ATPASE ACTIVITY

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ABSTRACT

The sarcoplasmic reticulum was isolated and characterized in the pure form from normal caprine (*Capra hircus*) hearts. Under ischemic condition, fatty acids like palmitic acid, palmitoyl carnitine and oleic acid accumulate *in vivo*. These are known to have a detrimental effect on membrane components. The effects of simulated ischemia on the Ca^{2+} -ATPase were studied using these fatty acids *in vitro* at concentrations at which they occur *in vivo* in the ischemic heart. All three fatty acids inhibited Ca^{2+} -ATPase activity. At lower concentrations, palmitoyl carnitine was the most potent, followed by oleic acid while palmitic acid displayed the least potency. Electron microscopy studies with fatty acids showed morphological disruptions in SR vesicles. The decline in Ca^{2+} -ATPase activity could be attributed to the change in membrane morphology.

KEY WORDS

Sarcoplasmic reticulum, Palmitic acid, Palmitoyl carnitine, Oleic acid, Simulated ischemia

INTRODUCTION

The heart prefers fatty acids over carbohydrates and proteins as oxidisable substrates, mitochondrial oxidation accounting for about 60-70% of the total amount of ATP required by the heart (1). Under normal physiological conditions this preference is adequately satisfied. However, under ischemic conditions, a lack of blood supply deprives the heart of oxygen limiting the availability of the same for oxidative phosphorylation; fatty acid and carbohydrate oxidation decline and ATP production is impaired. A series of metabolic failures ensues (2, 3). As a result, free fatty acids in the ischemic heart increase almost six to seven fold (4).

Cardiac function is influenced by changes in lipid composition of the cellular and subcellular membranes of myocardial cells. A modification in structure of cardiac membranes could produce significant abnormalities in cardiac function viz. mobility and conformation of intrinsic membrane proteins (3).

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The combined effect of lipid abnormalities finally contributes to a decline in myocardial contractility; the arrhythmias and eventual cell death that follow coronary artery occlusion ensue. Lipid abnormalities have been firmly established as a major factor leading to ischemic heart disease since the past couple of decades. Fatty acids are released from ischemic cardiac muscle due to activation of phospholipase A2 (5). Fatty acid accumulation in concentrations above normal is a hallmark of ischemia. Their effect interferes with the normal function of the heart and the SR in particular. Palmitic acid causes a reduction in the phospholipid content of SR membranes as observed from freeze fracture electron micrographs. In increasing concentrations, palmitic acid causes the SR membranes to undergo fusion with eventual breakdown of their vesicular structure. Palmitoyl carnitine produces marked changes in the structure and function of the sarcoplasmic reticulum and, at increasing concentrations, is believed to inhibit key membrane-bound enzymes, the most important being the marker enzyme Ca^{2+} -ATPase (6). Oleic acid, by disrupting the morphology of the membrane vesicles, causes permeability changes leading to the inhibition of ATP-induced calcium sequestration, in effect altering the activity of the marker enzyme Ca^{2+} -ATPase (7).

Despite the fact that *in vivo* studies are far superior and several times more comparable to human studies, *in vitro* studies have been widely carried out over the last few decades. Simulated

ischemia is a condition wherein the cellular and subcellular environment is reproduced *in vitro* to study the debilitating effects of several metabolites on isolated membranes, cells and their components. The goal of the present study is to establish a correlation between ischemia and the degree of impairment of the enzyme Ca^{2+} -ATPase *in vitro*.

MATERIALS AND METHODS

The SR was isolated from the heart by a modified method of Feher and Davis, 1991 (8).

SDS-PAGE - Individual aliquots of 50 μ l of the SR sample containing 50-60mg were treated with 5 μ M, 20 μ M and 50 μ M palmitic acid (PA); 10 μ M, 20 μ M and 40 μ M palmitoyl carnitine (PC); 6 μ M, 12 μ M and 16 μ M oleic acid (OA) were run on a 10 % SDS polyacrylamide gel. One untreated aliquot was maintained as the control. 50mg pre-treated SR samples were loaded per well. Wide range molecular weight marker from Sigma and BSA - Fraction V (Sigma) were used as standards.

Enzyme Assays - Individual aliquots of 50 μ l of the SR sample containing 50-60mg were treated with 5 μ M, 10 μ M, 20 μ M and 50 μ M palmitic acid (PA); 5 μ M, 10 μ M, 20 μ M, 40 μ M and 60 μ M palmitoyl carnitine (PC); 6 μ M, 12 μ M and 16 μ M oleic acid (OA) individually for 20 minutes at 37°C. One untreated aliquot was maintained as the control. Ca^{2+} -ATPase activity (SR marker enzyme) was determined for both 'untreated' and 'treated' SR aliquots at 37°C by measuring P_i production (9).

Electron Microscopy - PA, PC and OA were added individually to nine separate tubes of 30 μ l Buffer marked 'PA-5 μ M', 'PA-20 μ M', 'PA-50 μ M', 'PC-20 μ M', 'PC-40 μ M', 'PC-60 μ M', and 'OA-06 μ M', 'OA-12 μ M', 'OA-16 μ M' respectively in concentrations as specified on each tube. The tubes were incubated at 25°C for a period of 20 minutes. The suspension was then centrifuged at 25,000 rpm in an SW 40 swing out rotor for 30 minutes. The pellet obtained was dispensed in glutaraldehyde solution and stored at 4°C for a maximum of 2 hours prior to further treatment for EM viewing. SR samples for thin sections were fixed in cold 3% glutaraldehyde solution for 2 hours and treated with osmium tetroxide. After dehydration and embedding, thick sections were cut on an LKB Ultratome V, dried on a hot plate (60°C), stained with 1% toluidine blue and observed under the light microscope. The prepared blocks were trimmed, ultrasections cut and placed on 300 mesh size copper grids. Contrast was enhanced by a double staining technique - alcoholic uranyl acetate and lead citrate. Grids were observed under a JEOL -1010 electron microscope at 80kV accelerating voltage.

RESULTS AND DISCUSSION

There was no change in the banding pattern or the intensity of the component proteins of fatty acid treated SR samples when compared with the normal control SR. In this study we observed that addition of palmitic acid, at concentrations of 5 μ M, 10 μ M, 20 μ M and 50 μ M, to SR preparations, caused a significant reduction in SR Ca^{2+} -ATPase activity. The activity declined further with an increase in PA concentration [Table IV]. Similarly, palmitoyl carnitine and oleic acid caused a decline in Ca^{2+} -ATPase activity [Table V, VI]. 5 μ M resulted in 21% loss of activity while 10 μ M and 20 μ M palmitic acid reduced Ca^{2+} -ATPase activity by 33% and 32% respectively. 50 μ M yielded a loss of 65% enzyme activity [Table I]. 5 μ M palmitoyl carnitine resulted in a 40% decrease in enzyme activity. There was a 54% decline seen at 10 μ M, a 45% drop in activity was seen at 20 μ M and a 72% loss of activity at

Table I: Effect of varying concentrations of PA on Ca^{2+} -ATPase activity

	Table (a)	
	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PA (20 μ M) TREATED SR (n = 10)	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PA (50 μ M) TREATED SR (n = 10)
MEAN $\pm$ SEM	1.78 $\pm$ 0.235	3.65 $\pm$ 0.424
mmoles of P_i / mg protein / min		
P	<0.001	<0.001

	Table (b)	
	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PA (5 μ M) TREATED SR (n = 4)	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PA (10 μ M) TREATED SR (n = 4)
MEAN $\pm$ SEM	2.68 $\pm$ 0.064	4.21 $\pm$ 0.662
mmoles of P_i / mg protein / min		
P	<0.001	<0.01

40 μ M and 60 μ M each [Table II]. Oleic Acid at a concentration of 6mM inhibits enzyme activity by 32% and at 12 μ M the enzyme activity declines by 63%. 16 μ M showed a decline of 72% [Table III]. The Student's Paired t-Test has been employed to determine the statistical significance of the effect of fatty acids on the SR Ca^{2+} -ATPase. The enzyme activity was compared as the average difference between individual fatty acid-treated groups and normal (untreated) groups.

Table II: Effect of varying concentrations of PC on Ca^{2+} -ATPase activity

Table (a)		
	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PC (20 μM) TREATED SR (n = 10)	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PC (40 μM) TREATED SR (n = 10)
MEAN $\pm$ SEM mmoles of Pi / mg protein / min	4.60 $\pm$ 0.45	7.41 $\pm$ 0.74
P	<0.001	<0.001

Table (b)			
	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PC (5 μM) -TREATED SR (n = 4)	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PC (10 μM) -TREATED SR (n = 4)	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and PC (60 μM) -TREATED SR (n = 4)
MEAN $\pm$ SEM	5.17 $\mu\text{moles of Pi /}$ mg protein / min $\pm$ 0.72	6.83 $\mu\text{moles of Pi /}$ mg protein / min $\pm$ 0.516	9.15 $\mu\text{moles of Pi /}$ mg protein / min $\pm$ 0.592
P	<0.01	<0.001	<0.001

Ischemia has been found to profoundly alter the function of intrinsic and transmembrane systems (3). Fatty acids are one of many characteristic biochemical markers of ischemia as they accumulate in high concentrations within the myocardium in this diseased state. The effects of fatty acids in vitro were therefore studied to assess the activity of Ca^{2+} -ATPase activity and the structure of the membrane as a whole in the ischemic myocardium. Although different fatty acids have been known to accumulate during ischemia, the present study focuses on the effect of three distinctly different fatty acids on the SR, namely, saturated fatty acids (Palmitic Acid $\text{C}_{15}\text{H}_{31}\text{COOH}$), long chain acyl carnitines (Palmitoyl L-Carnitine Chloride $\text{C}_{23}\text{H}_{45}\text{NO}_4\cdot\text{HCl}$) and unsaturated fatty acids (Oleic Acid $\text{C}_{17}\text{H}_{33}\text{COOH}$). Fatty acid concentrations used are well in the range that is found under ischemic conditions and used widely in animal studies (10, 11). *We characterised the fatty acid-treated vesicles by (a) SDS-PAGE to visualise any change in density of individual protein components, (b) spectrophotometric analysis to quantify the change Ca^{2+} -ATPase activity and (c) electron microscopy to determine structural changes in membrane bilayer as the cause of an altered ATPase activity.*

Correlating the spectrophotometric analysis of Ca^{2+} -ATPase to the results of SDS-PAGE, we obtained a direct confirmation that the decline in ATPase activity is not due to any loss of

Table III : Effect of varying concentrations of OA on Ca^{2+} -ATPase activity

Table (a)		
	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and OA (6 μM) TREATED SR (n = 10)	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and OA (12 μM) TREATED SR (n = 10)
MEAN $\pm$ SEM mmoles of Pi / mg protein / min	1.30 $\pm$ 0.077	2.52 $\pm$ 0.335
P	<0.001	<0.001

Table (b)	
	Ca^{2+} -ATPASE ACTIVITY OF NORMAL and OA (16 μM) -TREATED SR (n = 4)
MEAN $\pm$ SEM mmoles of Pi / mg protein / min	9.16 $\pm$ 0.30
P	<0.001

Table IV: Summary of effect of PA on Ca^{2+} -ATPase activity

Table (a)			
Parameters	Groups		
	Normal (n = 4)	Palmitic Acid (5 μM) (n = 4)	Palmitic Acid (10 μM) (n = 4)
Mean $\pm$ SEM mmoles of Pi / mg protein / min	12.8 $\pm$ 0.48	10.12 $\pm$ 0.47	8.59 $\pm$ 0.75
Difference	-	Significant	Significant

Table (b)			
Parameters	Groups		
	Normal (n = 10)	Palmitic Acid (20 μM) (n = 10)	Palmitic Acid (50 μM) (n = 10)
Mean $\pm$ SEM mmoles of Pi / mg protein / min	5.65 $\pm$ 0.497	3.87 $\pm$ 0.584	2.00 $\pm$ 0.386
Difference	-	Significant	Significant

Ca^{2+} -ATPase pump units but due to a structural change in the lipid bilayer. The altered lipid environment prevents the pump from functioning optimally.

Incubation of the SR with the straight chain C16 saturated fatty acid **palmitic acid** caused the activity of Ca^{2+} -ATPase

Table V: Summary of effect of PC on Ca^{2+} -ATPase activity

Table (a)

Parameters	Groups			
	Normal (n = 4)	Palmitoyl Carnitine (5 μ M) (n = 4)	Palmitoyl Carnitine (10 μ M) (n = 4)	Palmitoyl Carnitine (60 μ M) (n = 4)
Mean $\pm$ SEM mmoles of Pi / mg protein/ min	12.8 $\pm$ 0.48	7.63 $\pm$ 0.97	5.97 $\pm$ 0.72	3.65 $\pm$ 0.30
Difference	-	Significant	Significant	Significant

Table (b)

Parameters	Groups		
	Normal (n = 10)	Palmitoyl Carnitine (20 μ M) (n = 10)	Palmitoyl Carnitine (40 μ M) (n = 10)
Mean $\pm$ SEM mmoles of Pi / mg protein / min	10.26 $\pm$ 0.497	5.66 $\pm$ 0.337	2.85 $\pm$ 0.485
Difference	-	Significant	Significant

to decline. A linear decline in Ca^{2+} -ATPase activity was observed with an increase in palmitic acid from 5 μ M to 50 μ M. **Palmitoyl carnitine**-treated vesicles like those treated with palmitic acid showed a linear decline in enzyme activity over a range of 5 μ M to 40 μ M, though lower concentrations caused a much higher decline in activity than palmitic acid at the same fatty acid concentrations. No significant decline was observed in the range of 5 μ M to 20 μ M. The linear decline in activity terminated in a plateau after 40 μ M palmitoyl carnitine

treatment. The sudden decline in activity shows that, unlike palmitic acid, the effects of this fatty acid are not gradual. Inhibition of Ca^{2+} -ATPase activity by the unsaturated fatty acid **oleic acid** in the range of 6-12 μ M was seen to be directly proportional to the concentration of oleic acid.

Our isolated SR samples treated with **palmitic acid** showed different structural alterations depending on the concentration of the fatty acid. At 5 μ M, there was no change in the structure of the vesicle as compared with the electron micrographs of normal SR (figure 1). A fusion of vesicles was seen at 20 μ M

Table VI: Summary of effect of OA on Ca^{2+} -ATPase activity

Table (a)

Parameters	Groups		
	Normal (n = 10)	Oleic Acid (06 μ M) (n = 10)	Oleic Acid (12 μ M) (n = 10)
Mean $\pm$ SEM	4.03 mmoles of Pi / mg protein / min $\pm$ 0.395	2.73 mmoles of Pi / mg protein / min $\pm$ 0.216	1.51 mmoles of Pi / mg protein / min $\pm$ 0.485
Difference	-	Significant	Significant

Table (b)

Parameters	Groups	
	Normal (n = 4)	Oleic Acid (16 μ M) (n = 4)
Mean $\pm$ SEM	12.8 $\pm$ 0.48 mmoles of Pi / mg protein / min	3.64 $\pm$ 0.27 mmoles of Pi / mg protein / min
Difference	-	Significant

Figure 1. EM OF NORMAL (i.e. UNTREATED)
CAPRINE CARDIAC SR SAMPLES (n=6) $\times$ 16000

while the fusion was more pronounced at 50 μ M (figure 2). Hence the drop in activity at low concentrations (5 μ M) is a result of two factors: (a) formation of calcium-palmitate aggregates (5) due to negatively charged palmitic acid at or near the surface of the phospholipid bilayer and (b) its incorporation into the bilayer without altering the spatial arrangement of the phospholipids. These conditions would result in the negatively charged palmitic acid forming a calcium pool independently and blocking both the availability of calcium to the ATPase pump and its subsequent infiltration into the

EM of caprine cardiac SR samples
treated with 20 μ M PA (n=6) x16,000

**Figure 2. EFFECT OF PALMITIC ACID ON THE SR
MEMBRANE STRUCTURE**

SR lumen (12). There is also a possibility of palmitic acid interacting directly with the active site of the enzyme (10). Other studies show a fusion of vesicles at 20 μ M resulting in a loss of phospholipid bilayer structure hence loss of fluidity and subsequent breakdown of the microenvironment suited to optimal ATPase activity (6). Electron micrographs of SR samples treated with palmitic acid in the range of 20-50 μ M show a loss of bilayer vesicular structure. Our study indicates a direct effect of palmitic acid at >20 μ M on the enzyme conformational structure resulting in a partial loss of activity. This deprives the ATPase enzyme of the suitable phospholipid environment necessary for its function (seen as a decline in activity).

Long chain acyl carnitines (LCAC) are amphipathic in nature allowing them to get incorporated into membranes and disrupt the phospholipid bilayer with subsequent functional alterations of membrane proteins (11). From our electron micrographs of

EM of caprine cardiac SR samples
treated with 40 μ M PC (n=6) x 40,000

**Figure 3. EFFECT OF PALMITOYL CARNITINE ON THE SR
MEMBRANE STRUCTURE**

EM of caprine cardiac SR samples treated with
06 μ M OA (n=6) x16,000

**Figure 4. EFFECT OF OLEIC ACID ON THE SR
MEMBRANE STRUCTURE**

SR vesicles treated with varying concentrations of **palmitoyl carnitine** (figure 3), we observed an incorporation of palmitoyl carnitine into the phospholipid bilayer leading to disruption of the vesicles. Palmitoyl carnitine at higher concentrations favours formation of multilamellar structures as is evident in figure 3. The degree of incorporation of **oleic acid** increases with an increase in fatty acid concentration as seen in figure 4. At 16 μ M, however, the vesicles are no longer markedly

defined and an eventual breakdown of their vesicular structure is imminent. From our electron micrographs it appears as though incorporation of the cis-unsaturated fatty acid, oleic acid (concentrations of 12 μ M and below), causes conformational changes in ion channel proteins within the membrane. This inhibits the ion movements responsible for excitation in the normal myocardium. An alteration in membrane thickness could also accompany these changes. Higher concentrations of oleic acid increase Ca²⁺ permeability of the SR and can disrupt the membrane structure (3).

The effects of exogenously added fatty acids on the Ca²⁺-ATPase observed in this study may occur because of (i) insertion into the bilayer, altering lipid-lipid and lipid-protein interactions, (ii) displacement of Ca²⁺ ions from the negatively charged binding sites on the membrane phospholipid heads, (iii) interaction with an active site in the hydrophobic core of the enzyme, (iv) influence on ion permeability and (v) alteration of membrane fluidity. Amphiphilic compounds in high concentrations are well known in perturbing membrane structure, ion transport and ATPase activity in both sarcoplasmic reticulum and sarcolemmal membranes (10). Our study demonstrated the wide disparities between the potencies of fatty acids with different chain lengths and degrees of saturation with respect to Ca²⁺-ATPase activity. At lower concentrations, palmitoyl carnitine is the most potent, followed by oleic acid while palmitic acid displays the least potency.

From this study it is evident that *in vitro* ischemic conditions have a detrimental effect on both the SR membrane and SR Ca²⁺-ATPase activity. However, this study encompasses the effect of only three fatty acids. *In vivo*, the amphiphilic compounds that accumulate in and around the myocardial cells during ischemia are structurally diverse and include fatty acids of variable chain and saturation as well as their carnitine and coenzyme A esters (10). We conclude that the combined accumulation of these metabolites could produce an additive effect leading to cardiac mechanical dysfunction seen in the ischemic heart.

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